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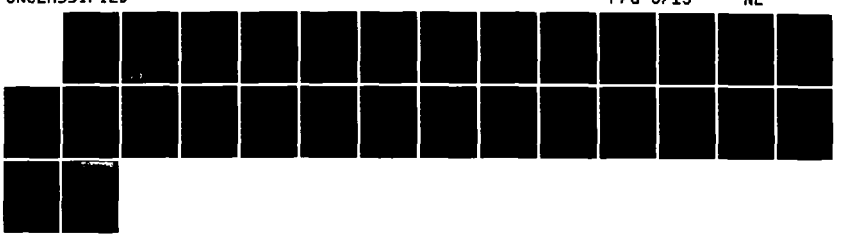

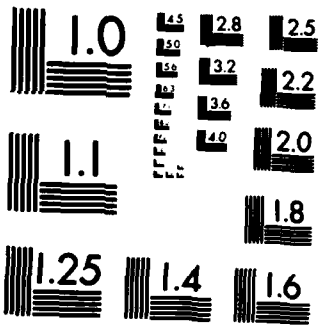

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ARMY 011581-A-03

AN INVESTIGATION OF MEMBRANE-ENCAPSULATED TRYPANOCIDES

ANNUAL REPORT

KARL J. HWANG

January 15, 1981

Supported by

U. S. ARMY MEDIGAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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This progress report describes our research in studying the in vivo properties of liposomes as a potential drug carrier for trypanocidal agents. The report contains four sections. The first section discusses the effect of chemical composition, size, and concentration on the degradation of liposomes in the liver of a mouse. The second section describes the effects of various factors on the clearance of liposomes in the blood of mice. The third section is a report on the development of methods of preparing various types of

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liposomes. The fourth section gives the detail in investigating the methodology of loading $^{111}In^{3+}$ and $^{67}Ga^{3+}$ to liposomes in a controlled manner.

In111(3+) and Ga67(3+)

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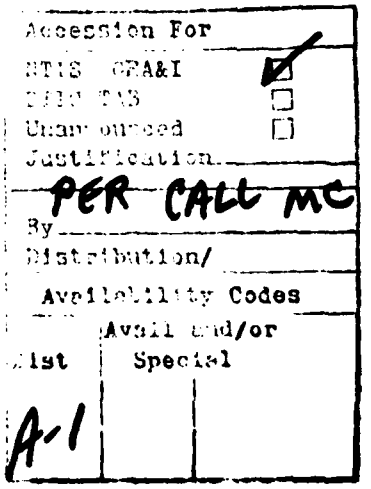
SUMMARY

This progress report describes our research in studying the degradation of liposomes in tissues and the factors affecting the blood clearance of liposomes. The progress report is divided into four sections. In the first section, the effects of chemical composition, size and concentration on the degradation of liposomes in tissues are discussed. Our result indicated that both the composition and size of liposomes can affect the kinetics of the release of entrapped $^{111}In^{3+}$. With the same composition, the rate of the release of the encapsulated $^{111}In^{3+}$ from small unilamellar liposomes was found to be faster than that from large liposomes. In the case of small unilamellar liposomes, the kinetics of degradation of liposomes in the liver did not appear to be affected by the amount of injected dose of liposomes. The second section describes the clearance of sphingomyelin liposomes in the blood circulation. Our results suggested that the small unilamellar sphingomyelin/cholesterol (2/1; M/M) liposomes can get an access to the intercellular space. This type of liposome may be used as a potential carrier of therapeutic agents against intercellular parasites. The third section is a report on the development of methods for preparing various types of liposomes for future investigation of how various factors may affect the degradation of liposomes. The fourth section gives the detail in investigating the methodology of loading $^{111}In^{3+}$ and $^{67}Ga^{3+}$ to liposomes in a controlled manner. Our result indicated that two new ionophores, namely, acetylacetone and 8-hydroxyquinoline sulfate may become useful agents to load a high level of $^{111}In^{3+}$ to liposomes for studying the modes of degradation of liposomes in tissues by the technique of perturbed angular correlation of gamma radiation.

FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.



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This annual report describes our research progress in the study of the degradation of liposomes in tissues, and the prolongation of the clearance time of liposomes in the blood circulation, during the period from January 1, 1980, to December 31, 1980. The research activity can be roughly categorized into four different areas. The first area discusses the effects of chemical composition and size of liposomes on the degradation of liposomes in tissues. The second area describes the effects of various factors on the clearance of liposomes in the blood of mice. The third area is in the development of methods of preparing various types of liposomes, and the fourth area is in the investigation of loading $^{111}In^{3+}$ and other trivalent cationic radionuclides to liposomes in a controlled manner. The objective of the last two areas is to develop methodologies for studying the degradation of liposomes in tissues by the techniques of perturbed angular correlation of gamma radiation (PAC). The research progress in these four areas can be summarized as follows:

I. Degradation of Liposomes in Tissues -- Effect of Chemical Composition, Size and Concentration.

Our research in this area has been directed primarily toward the comparison of the degradation of five types of liposomes in the livers of mice. A limited work has also been carried out in studying the degradation of liposomes in other tissues. These five types of liposomes were: (1)spingomyelin (SM): cholesterol (CH) (2/1; M/M) small unilamellar liposomes prepared by probe sonication; (2) distearoyl phosphatidylcholine (DSPC): CH (2/1; M/M) small unilamellar liposomes prepared by probe sonication; (3) SM:CH:dipalmitoyl phosphatidylethanolamine (DPPE) (57/33/10; M/M/M) small unilamellar liposomes prepared by probe sonication; (4) SM:CH (2/1; M/M) multilamellar liposomes prepared by extruding bath-sonicated, multilamellar liposomes through a polycarbonate membrane filter of 0.2μ pore size; and (5) SM: CH (2/1; M/M) bath-sonicated liposomes that had dimensions larger than the exclusion limit of a Sepharose 4B column, but smaller than that of a Sepharose 2B column.

Our initial plan was to study the rate of the in vivo degradation of liposomes in the liver at three to four different concentrations of injected dosage for each type of liposome. This study was very time-consuming. Normally, it took 3-4 weeks to collect one set of data for one concentration of liposomes. During the course of this study, we realized that the study of the in vitro degradation of liposomes in the livers of mice at 37°C could be a faster and more efficient approach to determine the range of the concentration of liposomes to be injected for investigating the dose-dependency of liposomes on the hepatic degradation of liposomes in vivo. Therefore, most of our results were obtained from dissected livers incubated in Ringer solution supplemented with 10% fetal serum and antibiotics at 37°C after the administration of liposomes by intravenous injection, as described in the attached publication.

The results of our studies indicated that the rate of the hepatic degradation of SM:CH (2/1; M/M) unilamellar liposomes in vitro was not affected by a 4-fold increase of liposomal dosage injected to mice. However, we observed quite a variation of the rate constant in the in vitro hepatic degradation. Figure 1 depicts the variation of the kinetics of the degradation of the same type of SM:CH (2/1; M/M) unilamellar liposomes in ten different livers. The data in Figure 1a were obtained from liposomes in which the $^{111}In^{3+}$ was loaded by chloroform-extracted 8-hydroxyquinoline ($^{111}In^{3+}$) according to the method described in our annual report of last year (ARMY 01580-A-02, Page 10). The data in Figure 1b were obtained from liposomes in which the $^{111}In^{3+}$ was loaded by 8-hydroxyquinoline ($^{111}In^{3+}$) without chloroform extraction, as described in Section IV of this report. These results appeared to suggest that the variation

of the kinetics of degradation may be related to how liposomes were prepared each time, and perhaps to the lot number of lipids from suppliers, since the degradation of the same batch of liposomes in two different mice often had an identical kinetics of the release of encapsulated $^{111}In^{3+}$. Work is in progress to investigate this possibility.

In comparison with SM:CH (2/1; M/M) small unilamellar liposomes, DSPC:CH (2/1; M/M) small unilamellar liposomes were found to be more susceptible of releasing the entrapped $^{111}In^{3+}$, either in the presence of serum, or in the liver of a mouse. The in vitro degradation of three preparations of DSPC:CH (2/1; M/M) small unilamellar liposomes is depicted in Figure 2. The fast rate of the release of encapsulated $^{111}In^{3+}$ could be, in part, a result of the presence of the contaminant of 1,3-distearoyl-glycero-2-phosphocholine, as recently suggested by Ponpipom and Bugianesi (1). Further work will be needed to study the degradation of DSPC by using purified DSPC.

We also found that an addition of 10% molar fraction of dipalmitoyl phosphatidylethanolamine in SM:CH small unilamellar liposomes did not seem to affect the rate of release of the entrapped $^{111}In^{3+}$ significantly (Fig. 3). However, the kinetics of the release of entrapped $^{111}In^{3+}$ appeared to be markedly affected by the size, or perhaps by the lamellar structure, of liposomes. Figure 4 depicts the time course of the release of $^{111}In^{3+}$ in the outermost layer of SM:CH (2/1; M/M) multilamellar liposomes extruded through a polycarbonate membrane filter of 0.2μ pore size in a liver of a mouse at 37°C in vitro. Figure 5 depicts a similar in vitro hepatic degradation of the outermost lipid bilayer of SM:CH (2/1; M/M) bath-sonicated liposomes that are larger than the exclusion limit of Sepharose 4B, but smaller than that of Sepharose 2B. To simplify the comparison, $^{111}In^{3+}$ was loaded only to the outermost aqueous compartment of the multilamellar liposomes for the studies shown in Figures 4 and 5. Thus, the results shown in Figures 4 and 5 represent the release of $^{111}In^{3+}$ resulting from the degradation of the outermost lipid bilayer of multilamellar liposomes, as in the case of unilamellar liposomes. As can be seen, the time course of the release of encapsulated material from MLV is very much different from that from SUV.

The marked difference in the time course of the release of liposome-encapsulated materials between small unilamellar liposomes and multilamellar liposomes was unexpected and interesting. Further work will be needed to elucidate whether or not such difference is a result of the size, the laminar structure, or the combination of both. We are in the process of collecting data to investigate whether or not such biphasic character of the degradation of multilamellar liposomes in the livers of mice also occurs in vivo.

In addition to the degradation of liposomes in the livers of mice, a limited study has also been carried out in the time course of the release of $^{111}In^{3+}$ from small unilamellar SM:CH (2/1; M/M) liposomes taken up by other tissues other than liver and blood. Since it is impossible to remove all the blood from tissues, only qualitative information can be obtained. Without performing a detailed correction of the blood contribution in each organ and the geometry effect, our preliminary results suggested that liposomes taken up by other organs were degraded at about one half of the rate of the liposomes taken up by the liver.

II. Clearance of Liposomes in Blood Circulation.

Our research in this area has been directed toward the comparison of the volume of distribution of small unilamellar SM:CH (2/1; M/M) liposomes with that of the intact erythrocytes, and the investigation of how the liposomal dosage,

laminar structure and composition of sphingomyeline liposomes may affect their elimination half-life in the circulation. The reason for determining the volume of distribution of liposomes was that, in the study of the tissue distribution of SM:CH (2/1; M/M) small unilamellar liposomes in mice, we invariably found that about 30% of the injected liposomes disappeared from the circulation at 15 min. post-injection. The uptake of liposomes by the liver and the spleen can only account for 5-6%. This may suggest that liposomes can cross the epithelial lining of the blood vessels, giving a larger distribution volume than that of the erythrocytes.

Two approaches were adopted for studying the distribution volume of liposomes. One is by sacrificing mice shortly after the administration of liposomes; the other is by pulse-chase experiments using dual isotope techniques. Our results indicated that the distribution volume of SM:CH (2/1; M/M) small unilamellar liposomes was about 20% larger than that of the red blood cells. Conceivably, this extra 20% volume may represent the intercellular space. Furthermore, from the pulse-chase experiment, we found that the pools of liposomes in the erythrocyte volume and in the intercellular volume were exchangeable readily. The ability for the SM:CH unilamellar liposomes to get an access to the intercellular space suggests that this type of liposome could be used for carrying therapeutic agents against intercellular parasites.

In the study of the effect of liposomal dosage on the clearance, we found that the elimination half-life increased with increasing dosage of SM:CH (2/1; M/M) small unilamellar liposomes. The saturation level for the Balb/c mouse was found to be about 100 μg lipid per g of body weight (Fig. 6). The blood clearance time of small unilamellar SM:CH:DPPE (57/33/10; M/M/M) liposomes was found to be quite similar to that of small unilamellar SM:CH (2/1; M/M) liposomes. In contrast to small unilamellar SM:CH (2/1; M/M) liposomes, multilamellar SM:CH (2/1; M/M) liposomes, prepared by extruding through a polycarbonate membrane filter of 0.2μ in pore size, had a very short half-life of blood clearance, with about 80% of the injected dose of liposomes taken up by the liver at 15 minutes after intravenous administration.

Using dual isotopes of $^{111}In^{3+}$ and $^{67}Ga^{3+}$ as the respective encapsulated markers of SM:CH (2/1; M/M) and DSPC:CH (2/1; M/M) small unilamellar vesicles (SUV), our results indicated that the half-life of SM:CH (2/1; M/M) SUV was twice as long as that of DSPC:CH (2/1; M/M) SUV. The comparison of the clearance time of various types of liposomes is summarized in Figure 7. In comparing the size of unilamellar liposomes of SM:CH (2/1; M/M), DPPC:CH (2/1; M/M) and DSPC:CH (2/1; M/M), we found that the average diameter of DSPC liposomes was larger than that of SM or DPPC liposomes. Further work will be needed to investigate whether the differences in the half-life of clearance of these small unilamellar liposomes result from the difference in size, stability in the blood circulation, or other reasons.

III. Encapsulation of Small Unilamellar Vesicles by Multilamellar Vesicles and Preparation of Detergent-Removed Liposomes.

As shown in the first section of this report, the degradation of liposomes in the liver of a mouse is affected markedly by the size of liposomes. Conceivably, the lamellar structure of liposomes can also affect the degradation of liposomes. To address these questions, some of our research has recently been directed toward the development of methodologies for preparing bovine brain sphingomyelin and DSPC liposomes of varying size and lamellar structure, with special emphasis on the investigation of encapsulating small unilamellar vesicles (SUV) by multilamellar vesicles (MLV) and the preparation of detergent-removed liposomes of varying diameter.

The development of methods to encapsulate SUV by MLV will be useful for the preparation of a special type of liposome in which only the center core, and not the outer shell, of the onion-like spherule entraps $^{111}In^{3+}$. This type of liposome will be useful for investigating how the outer layers of a multilamellar liposome may affect the degradation of the inner layer.

To encapsulate a preformed SUV by MLV, two different approaches were tested. The first approach was by shaking, or brief sonicating, in a water bath of a suspension of SUV in a tube containing dried lipids of different compositions. The dried lipid was in the form of dried powder, granules, or thin film. The second approach was by injecting an ether solution of lipids into an aqueous suspension of SUV. In most cases, the complex of $^{111}In^{3+}$ with nitrilotriacetic acid (NTA), or 6-carboxyfluorescein alone, was encapsulated in the SUV as a marker. The formation of MLV was assessed by passing the final suspension of liposomes through a Sepharose 4B or 2B column. The criteria of assessing a successful encapsulation of SUV by MLV were: (i)the association of the SUV-encapsulated marker with MLV: (ii) the ability to release the SUV-encapsulated marker from MLV upon treatment of detergent; and (iii) the presence of the intact preformed SUV in the fractions of MLV eluted from a Sepharose 4B or 2B column.

Our results indicated that, in most cases, the small molecular markers encapsulated in a preformed SUV tended to leak out during the process of bath sonication or injection of a large volume of lipid solution in ether into the aqueous suspension of SUV. It appeared that the time of bath sonication and the volume of injected ether were critical in inducing the release of the encapsulated small molecular marker. Because of this complication, the estimation of the percentage of a preformed SUV encapsulated in MLV was difficult. However, in certain cases, we found that the encapsulation of bovine brain sphingomyelin:cholesterol (2/1; M/M) SUV by soybean phospholipids MLV was negligible. This suggests that the encapsulation of a preformed SUV by MLV may be related to the compatibility of lipids. Further work will be needed to investigate the feasibility of encapsulating a preformed SUV by MLV by using a more gentle way of preparing MLV or by using a macromolecular marker.

In the study of preparing liposomes of controlled dimensions, our research effort has been concentrated on the preparation of MLV of 0.22μ in diameter by extruding MLV through a series of polycarbonate membrane filters from 1μ to 0.2μ in pore size, and the preparation of SUV of 340-1280 Å in diameter by removing detergent from a mixture of bile salt and phospholipids. These two types of liposomes can be prepared quite easily from egg phosphatidylcholine, as described in the literature. Since, from our previous study, the egg phosphatidylcholine liposomes are very leaky and unstable in the presence of serum (Page 11-12 of our annual report of 1979, ARMY 011580-A-02), our main effort was to prepare stable liposomes made from bovine brain sphingomyelin and disteroylphosphatidylcholine.

The results of our studies indicated that these two types of liposomes shared a common property -- a high tendency to adhere to resin materials of Sepharose, Sephadex, and Bio-Rad AG1X-8. As a result of such tight adhesion to resin materials, one often lost the entire liposome during the process of chromatography. In the case of membrane-extruded MLV, a repeat extrusion and annealing of liposomes, at a temperature above the gel → liquid crystalline phase transition temperature of the liposomes, were necessary to reduce the problem of adhesion. However, an occasional zero-recovery of liposomes during the process of gel filtration chromatography still

occurred without obvious reasons. Further work will be needed to establish a proper protocol to eliminate this problem.

In the study of preparing unilamellar liposomes of controlled sizes, liposomes were prepared by removing sodium deoxycholate from a mixture of phospholipids, cholesterol, and the bile salt, by dialyzing out the detergent in hollow fibers or by passing through a Sephadex G25 column (3-6). Our results indicated that the removal of detergent by passing through a Sephadex G-25 column was more effective and rapid than that by dialysis in hollow fibers The use of a constant-temperature jacketed column at a temperature above the temperature of the phase transition of the major lipid of the liposomes was required to ensure above 80% recovery of lipids. Furthermore, a high ratio of detergent to lipid (8 to 1 in molar ratio) was also necessary to ensure quantitative recovery of lipids, when cholesterol was included in the preparation of liposomes. The size of liposomes prepared by removing sodium deoxycholate from a lipid-detergent mixture appeared to be related to the composition of lipids. In the case of bovine brain sphingomyelin, a very small unilamellar vesicle of about 300 Å in diameter was formed. We are in the process of studying the ability of these detergent-removed liposomes to encapsulate various markers.

IV. Controlled Loading of $^{111}In^{3+}$ to Liposomes.

In order to study how various factors may affect the degradation of liposomes in tissues by the technique of PAC, it is important to be able to load a high level of $^{111}In^{3+}$ to the inner aqueous compartments of liposomes in a controlled and consistent manner. In our annual report of 1979, we described the procedure of extracting the complex of 8-hydroxyquinoline (8-HOQ) with $^{111}In^{3+}$ by chloroform for subsequent loading of $^{111}In^{3+}$ to liposomes with an efficiency of 90%. However, during the course of numerous loadings of $^{111}In^{3+}$ to liposomes for studying the degradation of liposomes in tissues, we found that many factors could affect the efficiency of loading $^{111}In^{3+}$ to liposomes, causing the problem of inconsistency in loading efficiency. Therefore, the procedure of loading has been gradually modified and evolved by changing various experimental conditions, or by using different lipid-soluble ionophores, since we reported the method of loading in our annual report of 1979.

To date, we have tracked down three major factors which can severely interfere with the efficiency of loading $^{111}In^{3+}$ to liposomes. The first problem was the loss of 8-HOQ by sublimation during the process of removing the chloroform from the extracted complex of 8-HOQ ($^{111}In^{3+}$) by evaporation at 85°C (Page 22 of our annual report of 1979). By monitoring the concentration of 8-HOQ with absorbance at 240 nm, we discovered that as much as 80-90% of the 8-HOQ, but not 8-HOQ ($^{111}In^{3+}$), was lost by sublimation during the process of evaporating chloroform at 68°C. The problem of losing 8-HOQ by evaporation was further compounded by the use of a very small amount of ethanol (25µl-50µl) to solubilize the dried 8-HOQ ($^{111}In^{3+}$), which formed a thin film and spread around a relatively large surface of the glass wall of a conical centrifugation tube. As a result of the use of an uncertain amount of 8-HOQ, the loading efficiency varied, depending upon the amount of leftover 8-HOQ in the tube after evaporation, and the volume of ethanol used in solubilizing the dried 8-HOQ ($^{111}In^{3+}$).

A modified loading procedure without chloroform extraction was, thus, developed to circumvent the problem of losing 8-HOQ. This modified loading procedure involved the direct addition of the mixture of $^{111}InCl_3$ and 8-HOQ in 5 mm sodium acetate buffered saline containing 7-70% ethanol to 10 times the volume of liposome suspension in the same buffer without ethanol. The results of this non-extraction loading procedure (Figs. 8,9) indicated that the loading efficiency depended very much upon the concentration of 8-HOQ in the solution, as well as the type of cation. It appeared that the optimal concentrations of 8-HOQ for a successful loading of $^{111}In^{3+}$ and $^{67}Ga^{3+}$ were very different. The exact mechanism is not certain at this point.

Normally, the complex $^{111}In^{3+}$ with the liposome-entrapped water-soluble chelating agent, nitriloacetic acid (NTA), has a rather fast tumbling rate as indicated by a high $<G_{22}(\omega)>$ value from the measurement of PAC. Our second problem was an apparent high loading efficiency (about 80 to 90%) but a low $<G_{22}(\omega)>$ value for the $^{111}In^{3+}$ associated with liposomes. The results of gel filtration chromatography indicated that virtually all the radioactivity of $^{111}In^{3+}$ in the loaded liposomes was firmly bound to liposomes and could not be removed by strong chelating agents, such as ethylenediaminetetraacetic acid (EDTA). This suggested that the loaded $^{111}In^{3+}$ ions were immobilized in the EDTA-inaccessible region of the lipid bilayer of liposomes, or in the internal aqueous compartment of liposomes.

The problem of having $^{111}In^{3+}$ immobilized in liposomes will affect the sensitivity of our PAC study of the degradation of liposomes after being taken up by tissues. In an attempt to determine the causes of this problem, we investigated all the possible sources of contamination in the buffer solution, chemical reagents, lipids, resin materials, glassware, and the source of $^{111}In^{3+}$. Our results indicated that one of the most likely sources of contamination was the HCl used in purifying $^{111}In^{3+}$. The HCl used in purifying $^{111}In^{3+}$ was always treated with dithizone to remove excess heavy metalic ions in HCl. The dithizone treatment involved the repeat extraction of 6N HCl with 0.01% dithizone in chloroform. Previously, 0.1 N HCl was used to elute $^{111}InCl_3$ from the anion exchange resin AG1X8. More recently, 2N HCl has been used to increase the resolution of $^{111}InCl_3$, since the discovery of the overlap of the eluting profile of some contaminating cations in the crude $^{111}InCl_3$ with the eluting peak of $^{111}InCl_3$. The use of more HCl may have introduced an excess of dithizone in the purified $^{111}InCl_3$. Dithizone is very soluble in chloroform and not soluble in neutral aqueous solution. We suspected that the complex of dithizone with $^{111}In^{3+}$ might partition in the lipid bilayer of liposomes, immobilizing $^{111}In^{3+}$. A new procedure of removing heavy metalic cations from HCl by the method of isoprestical distillation was, thus, employed. Our results indicated that dithizone was the major contaminant interfering with the loading of $^{111}In^{3+}$ to liposomes.

The third factor which could interfere with the loading efficiency of $^{111}In^{3+}$ to liposomes was the problem of forming insoluble 8-HOQ micro-particulate upon adding the ethanol solution of 8-HOQ to aqueous buffer solution at low temperature. At first, this solubility problem was not obvious to us because of the use of a low concentration of 8-HOQ in the loading mixture, until we accidently found this phenomenon by making a large volume of loading mixture. Although the 8-HOQ micro-particulate could be redissolved in aqueous-ethanol mixture upon standing at room temperature, the transient low concentration of soluble 8-HOQ could result in the formation of insoluble indium hydroxide, which can only be resolubilized at very low pH 2. Thus, all the buffer solutions used in loading $^{111}In^{3+}$ to liposomes were warmed to room temperature to avoid the possibility of phase separation of 8-HOQ.

In the meantime, the search for lipid ionophores which have a good aqueous solubility was initiated. Two ionophores were found to be quite promising. The two ionophores were 8-hydroxyquinoline sulfate (8-HOQ-SO_4) and acetylacetone (AcAc). Our preliminary results indicated that the loading efficiency of $^{111}In^{3+}$ liposomes by these two water-soluble ionophores was very encouraging. The principle of loading $^{111}In^{3+}$ to liposomes by 8-HOQ-SO_4 is essentially the same as that by 8-HOQ alone. The only difference is that 8-HOQ-SO_4, which is a sulfate salt of 8-HOQ, can form a stable salt-complex in aqueous solution, whereas 8-HOQ alone is not water-soluble. As a result of this, the probability of having the problem of forming the insoluble 8-HOQ microparticulate in aqueous solution is less. All the experimental conditions developed for 8-HOQ were found to be compatible to 8-HOQ-SO_4.

On the other hand, acetylacetone is slightly more hydrophilic than 8-HOQ. We found that the complex of $^{111}In^{3+}$ with acetylacetone had a very stable $\langle G_{22}(\omega)\rangle$ of 0.68 ± 0.02. As in the case of 8-HOQ, the loading efficiency of $^{111}In^{3+}$ to liposomes by acetylacetone depended on the concentration of acetylacetone (Fig. 10). Bovine brain sphingomyelin:cholesterol (2/1; M/M) liposomes (SUV or MLV) loaded to $^{111}In^{3+}$ by acetylacetone were stable at 37°C in the presence of 50% serum for at least 2 days. Since acetylacetone is quite soluble in aqueous solution, we are in the process of testing the feasibility of using acetylacetone as a carrier to deliver $^{111}In^{3+}$ into the many inner aqueous compartments of multilamellar liposomes. In conjunction with the development of methods of encapsulating small unilamellar liposomes by multilamellar liposomes, acetylacetone may become a very useful ionophore to load $^{111}In^{3+}$ to liposomes for studying how the lamellar structure of a liposome may affect or control the release of liposome-entrapped agents in tissues.

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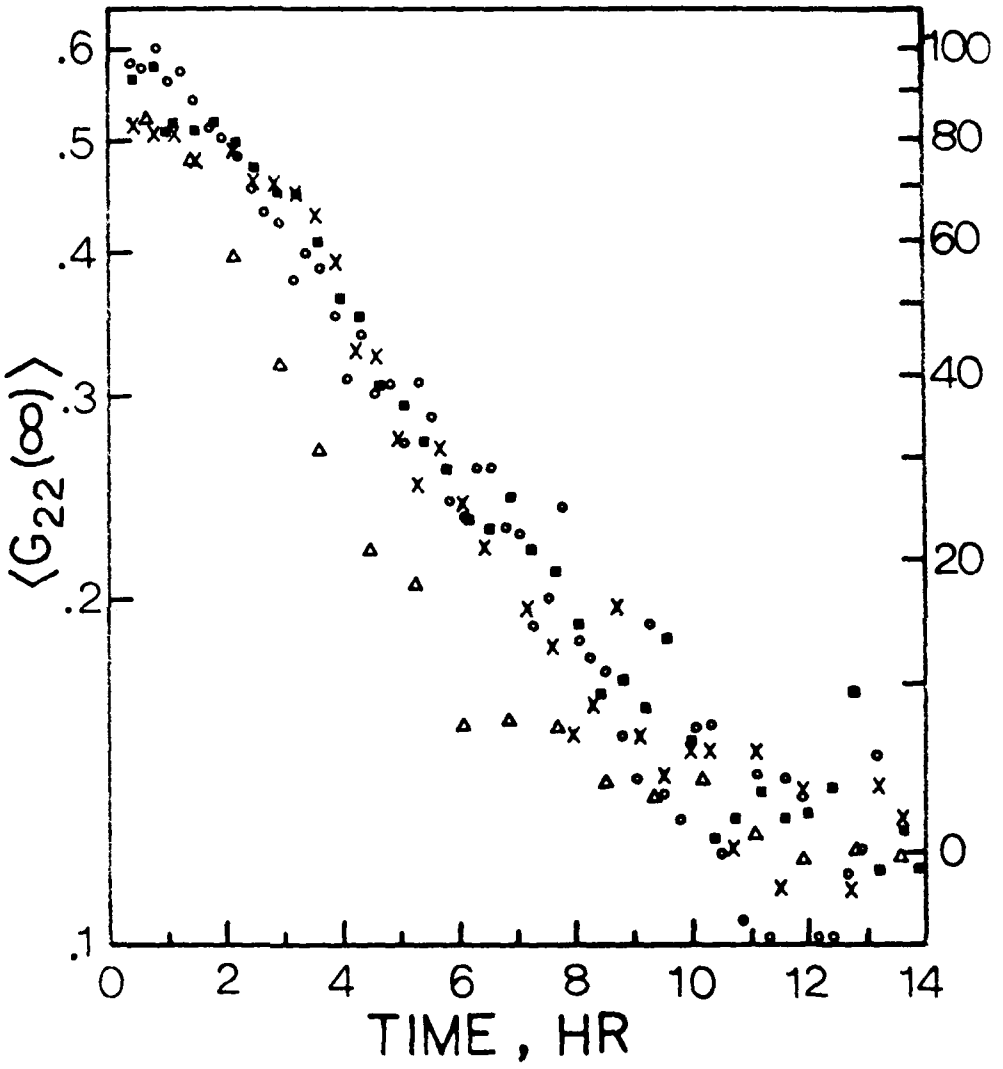


Figure 1a. Degradation of small unilamellar SM:CH(2/1;M/M) liposomes in the livers of 4 mice. The time course of the release of $^{111}In^{3+}$ from liposomes in the liver was monitored in vitro at 37°C. The scale on the right represents the percentage of intact liposomes remaining in the liver. The liposomes were loaded with $^{111}In^{3+}$ by chloroform-extracted 8-HQO ($^{111}In^{3+}$). (○) and (■) were from the same liposomes in two different livers.

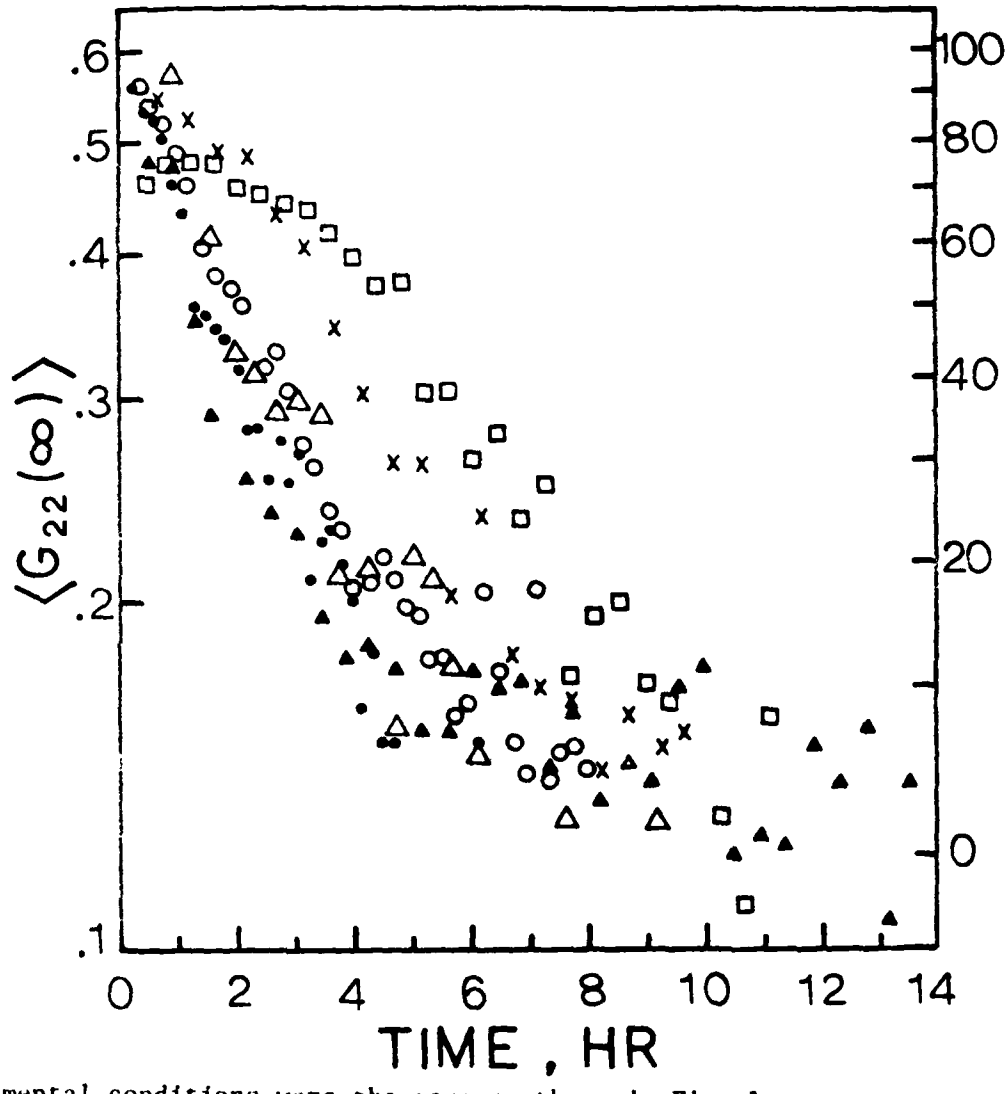


Figure 1b. The experimental conditions were the same as those in Fig. 1a, except that $^{111}In^{3+}$ was loaded to liposomes by 8-HOQ ($^{111}In^{3+}$) without prior purifying of the complex by chloroform extraction. (●) and (▲) were from the same liposomes in two different livers at an injected dose of 8 and 52 μg lipid/g tissue, respectively. (Δ) and (o) were from the same liposomes in two different livers at an injected dose of 90 and 224 μg lipid/g tissues, respectively. The kinetics of degradation of the liposomes subjected to ultracentrifugation at 1 0,000 g for 0 min (X) at an injected dose of 85 μg lipid/g tissue was comparable to that of the same liposomes subjected to centrifugation at 10,000 g for 3 min (□) at an injected dose of 99 μg lipid/g tissue.

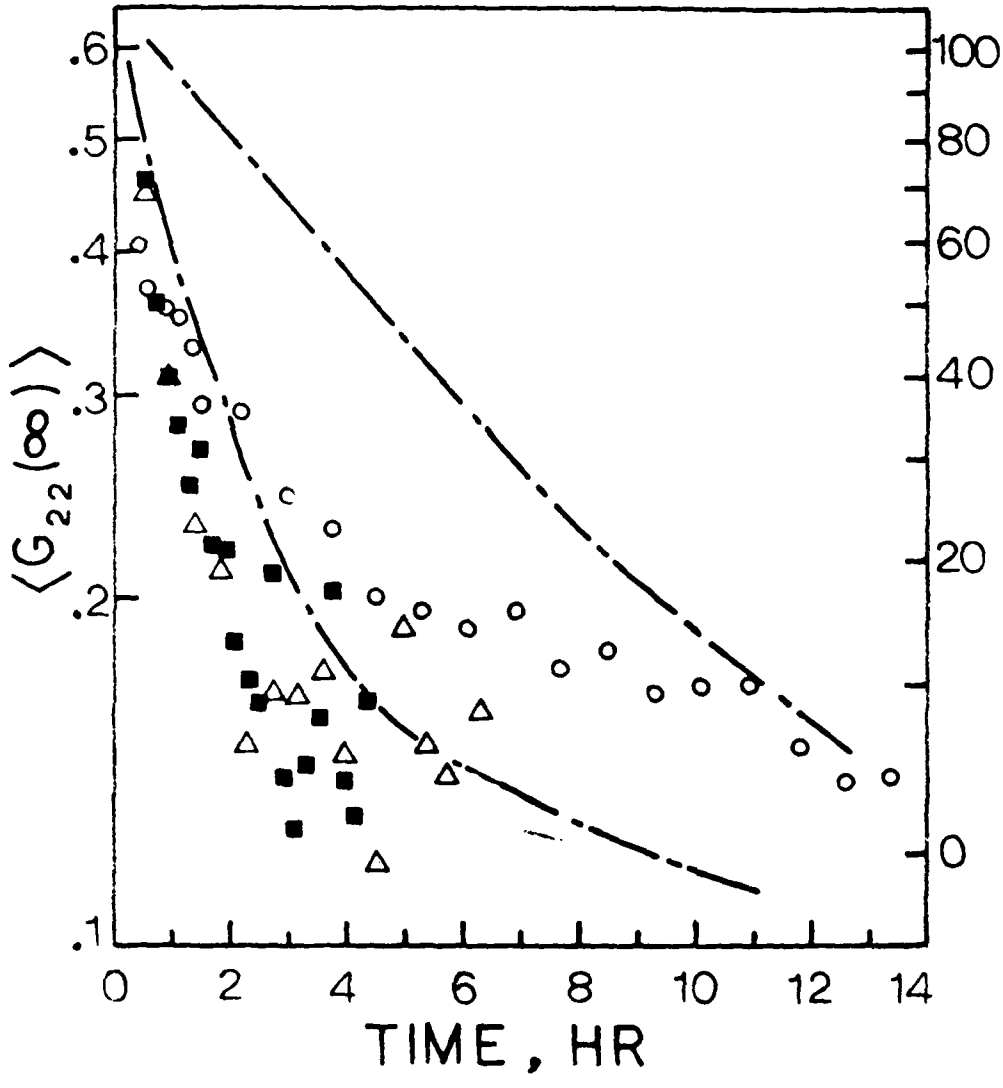


Figure 2. Degradation of small unilamellar DSPC:CH(2/1;M/M) liposomes in the livers of three mice. The experimental conditions were the same as that in Figure 1b. The area between the two dotted lines represents the region for SM:CH(2/1;M/M) small unilamellar liposomes shown in Fig. 1a and 1b.

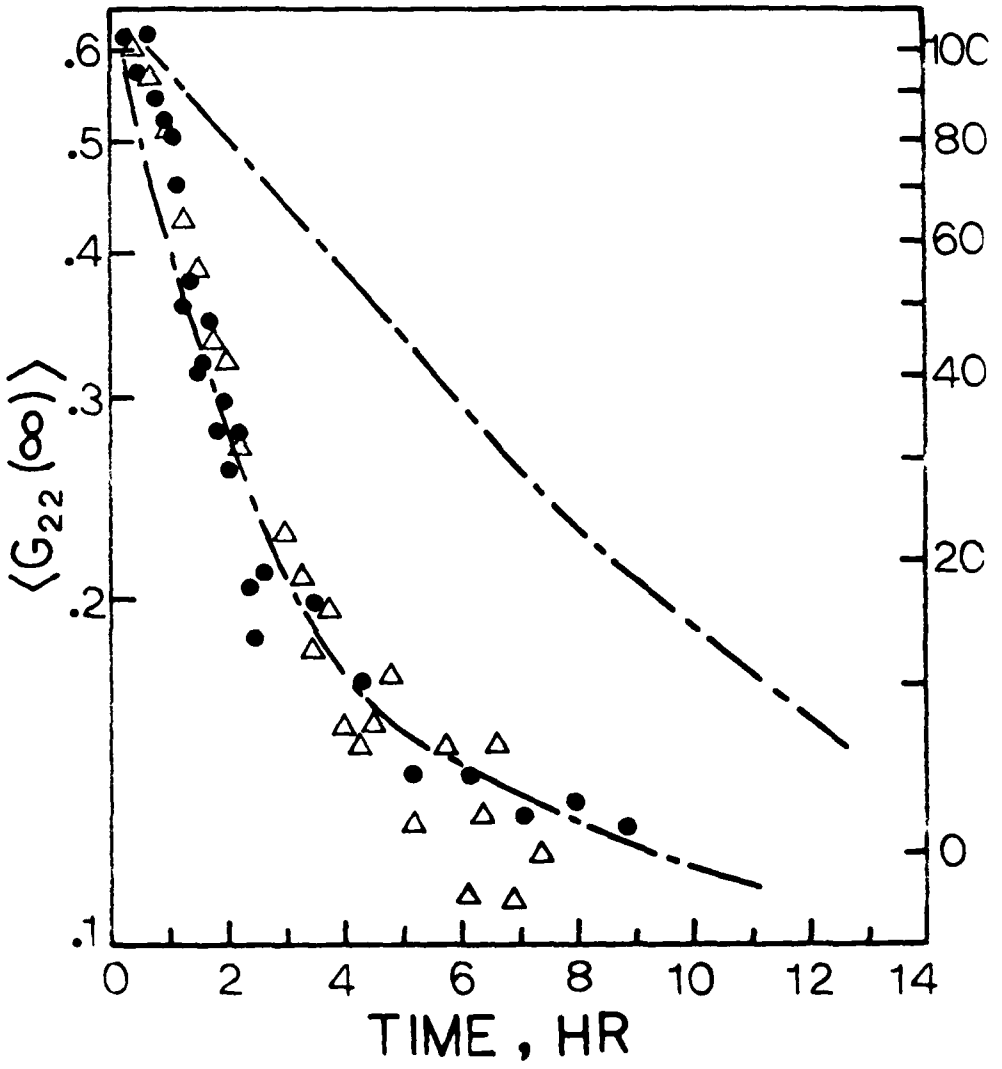


Figure 3. Degradation of small unilamellar SM:CH:DPPE(56/34/10;M/M/M) liposomes in the livers of two mice. The experimental conditions were the same as that in Fig. 1b. The area between the two dotted lines represents the region for SM:CH(2/1;M/M) small unilamellar liposomes shown in Fig. 1a and 1b.

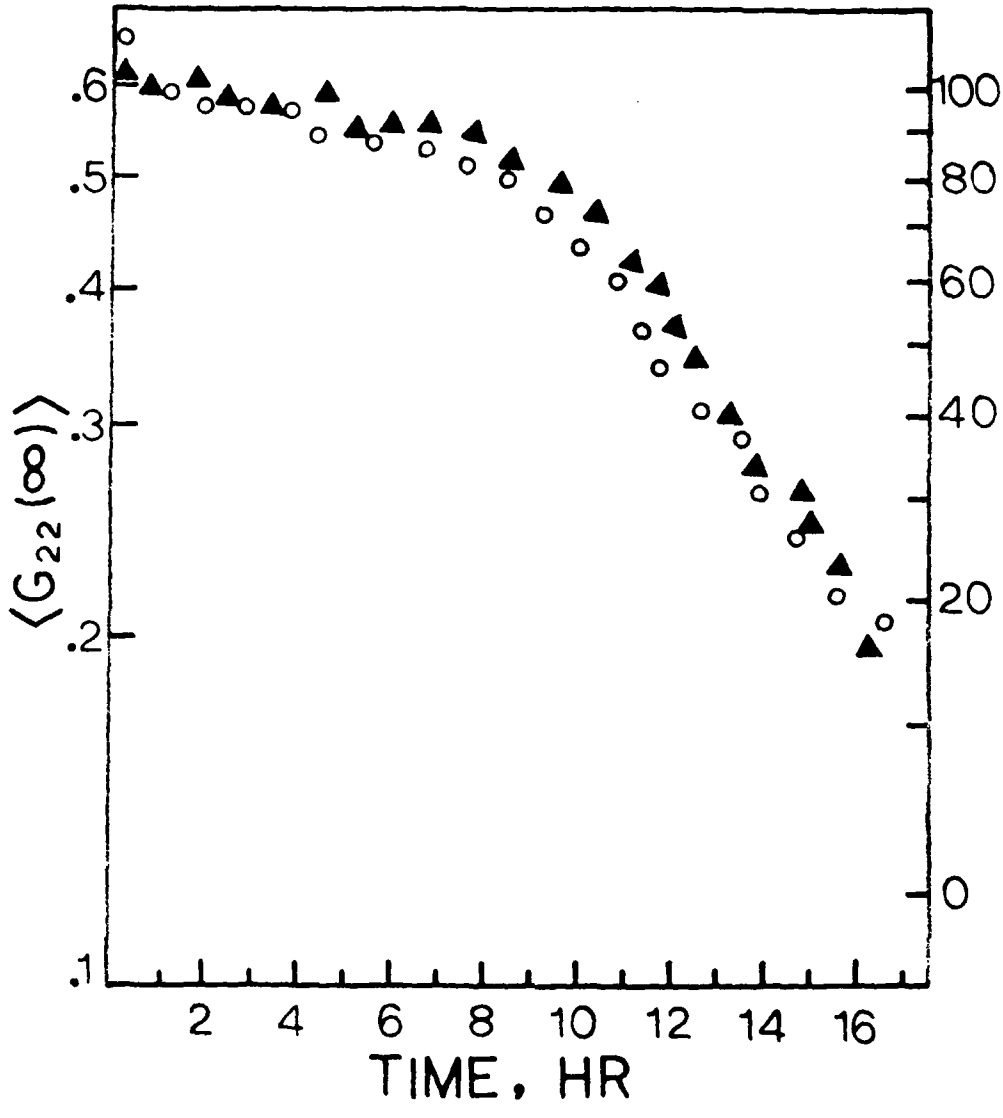


Figure 4. Degradation of membrane-extruded multilamellar SM:CH(2/1;M/M) liposomes in the livers of two mice. Liposomes were extruded through a series of polycarbonate membrane filters from 1.0 μ to 0.2 μ pore sizes at 60°C. The outermost aqueous compartment of the liposomes were loaded with $^{111}In^{3+}$ by 8-HOQ($^{111}In^{3+}$). Thus, the pattern of the release of $^{111}In^{3+}$ represents the breakdown of the outer surface of the multilamellar liposomes.

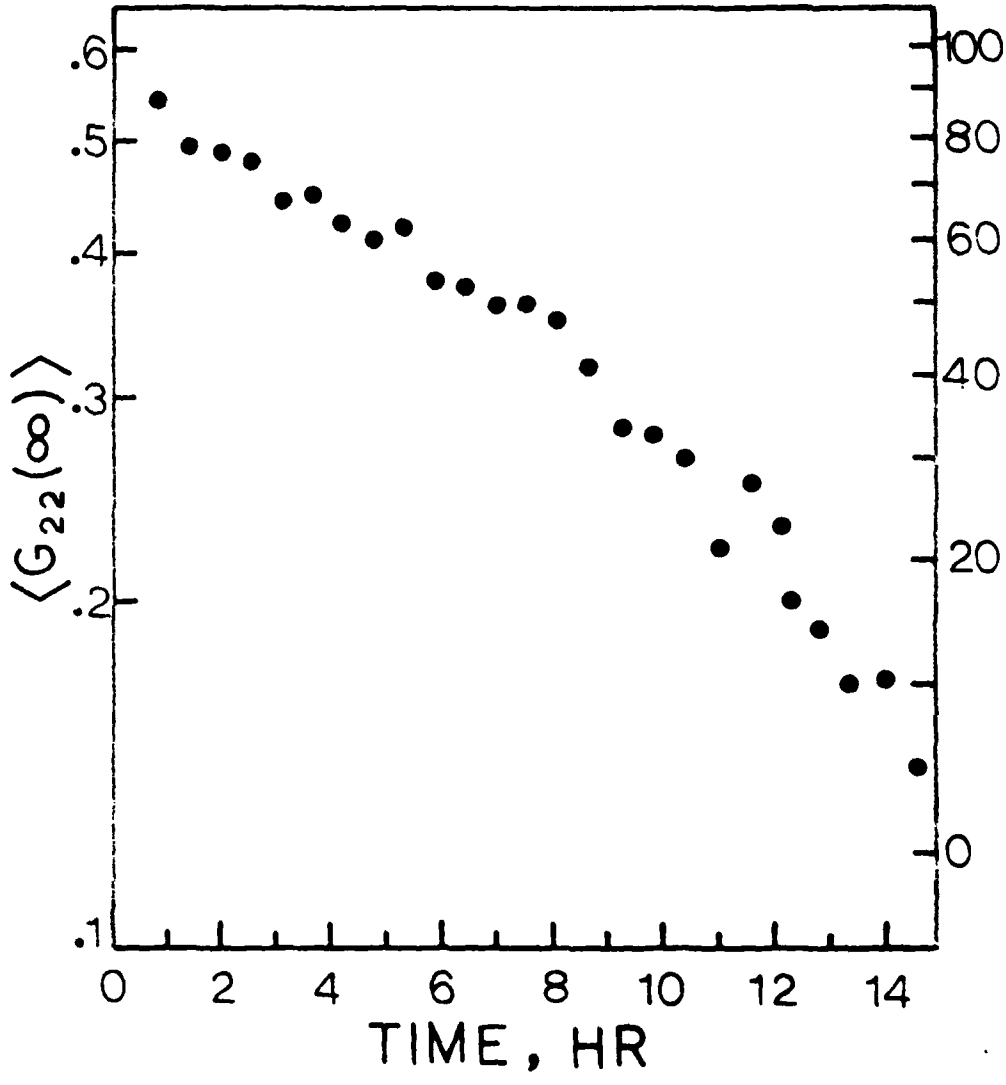


Figure 5. Degradation of bath-sonicated, intermediate, multilamellar SM:CH (2/1;M/M) liposomes in the liver of a mouse. The sizes of the liposomes were smaller than the exclusion limit of Sepharose 2B gel, but larger than that of Sepharose 4B gel. The time course of the release of $^{111}In^{3+}$ represents the degradation of the outer surface of multilamellar liposomes.

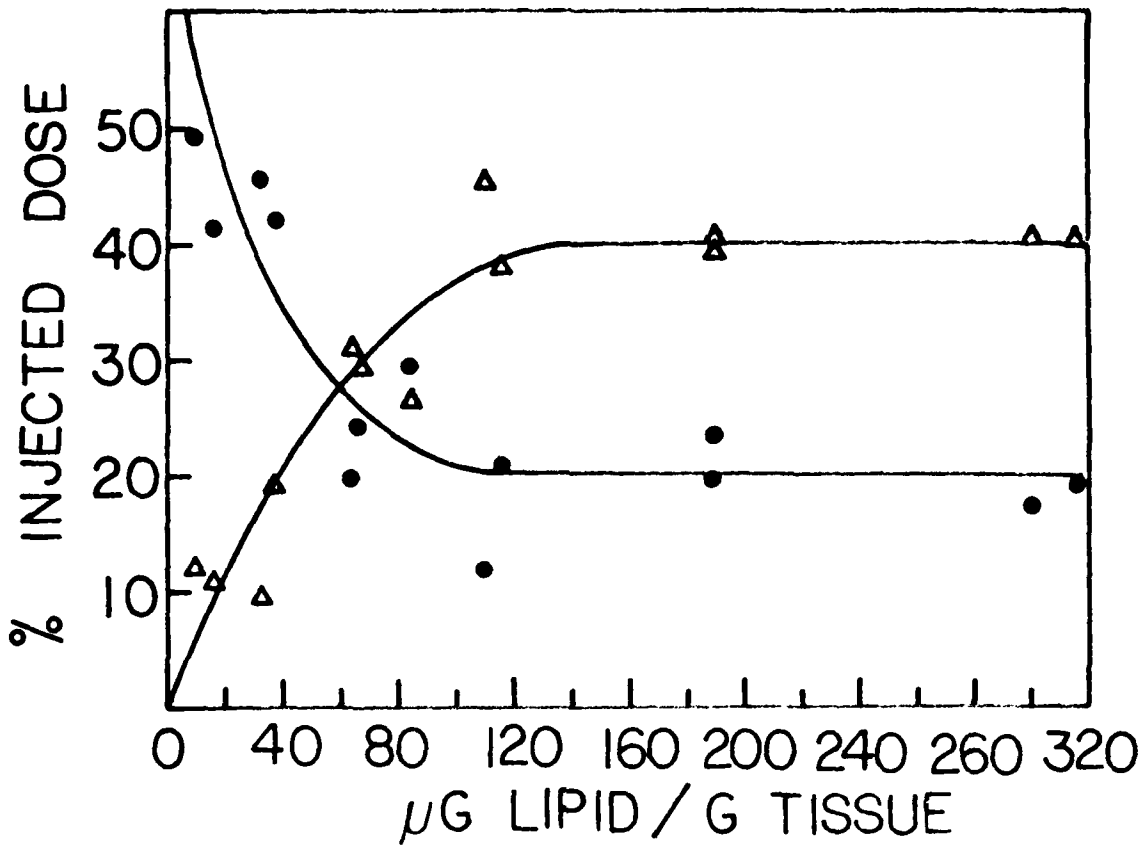


Figure 6. Effect of liposomal dosage on the blood clearance and hepatic uptake of small unilamellar SM:CH (2/1;M/M) liposomes. The percentages of injected dose in the blood (Δ) and in the liver (•) were determined at 23 hours after administering a varying amount of liposomal lipid per body weight through the tail veins of mice.

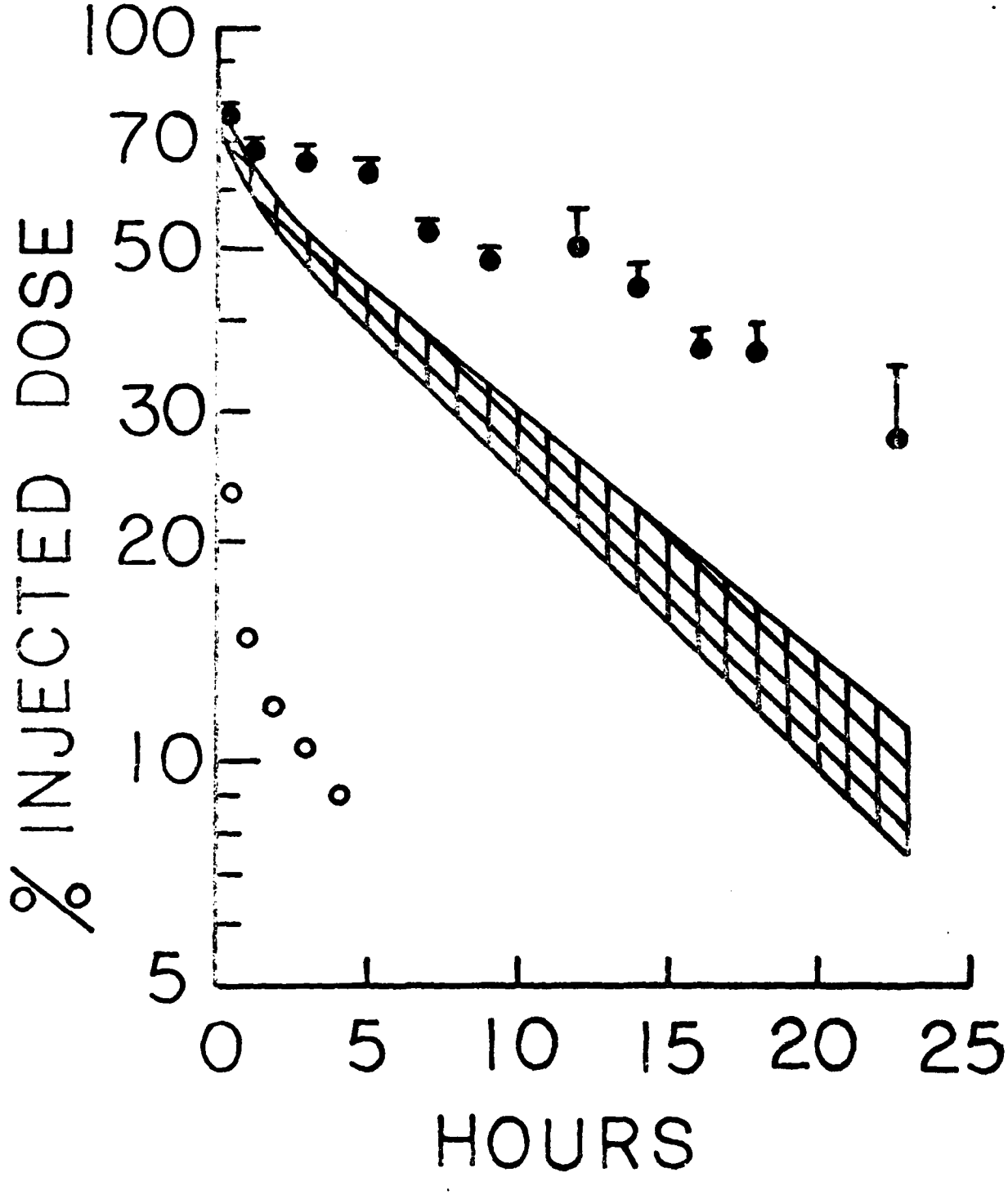


Figure 7. Blood clearance of various types of liposomes. The symbols are: small unilamellar SM:CH(2/1;M/M) liposomes (●), multilamellar SM:CH(2/1;M/M) liposomes (o), and the shaded area for small unilamellar DPPC:CH(2/1; M/M) and DSPC:CH (2/1;M/M) liposomes.

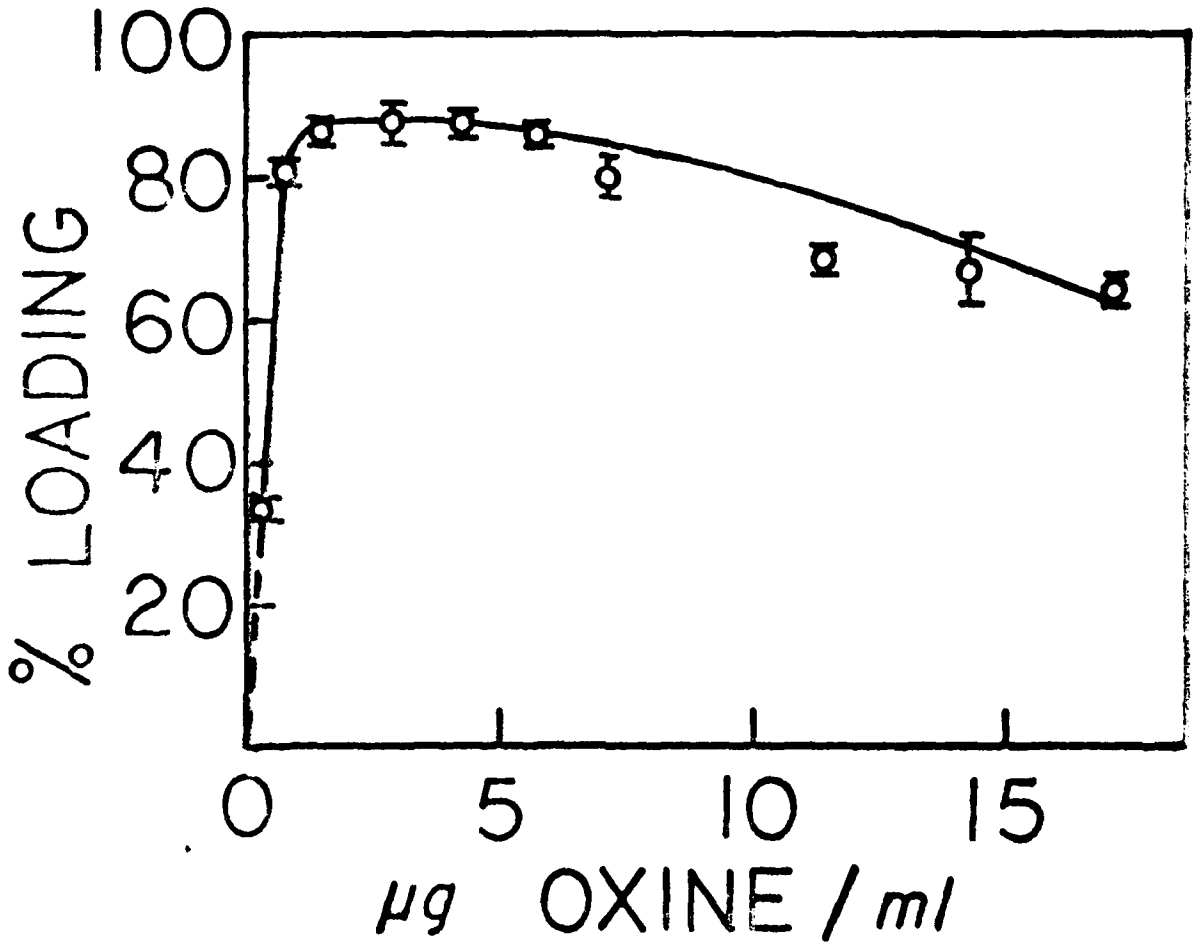


Figure 8. Dependence of the loading efficiency of $^{111}In^{3+}$ to liposomes on the concentration of 8-HOQ. The loading was carried out by adding 200-250 μl of a varying amount of 8-HOQ in 20% ethanol, 0.9% NaCl, 10 MM sodium acetate, pH 5.5 containing 1-10 μ Ci In-111 to 500 μl of DPPC unilamellar liposomes (5-8 mg lipid) in the same saline acetate buffer. The liposomes entrapped 0.106 M sodium phosphate, pH 7.4, 1mMNTA. The yield of loading was determined after 1 hr incubation at room temperature before removing the untrapped $^{111}In^{3+}$ by passing through a column (0.7 x 5 cm) of AG 1x8 (phosphate form). Each point is an average of two measurements.

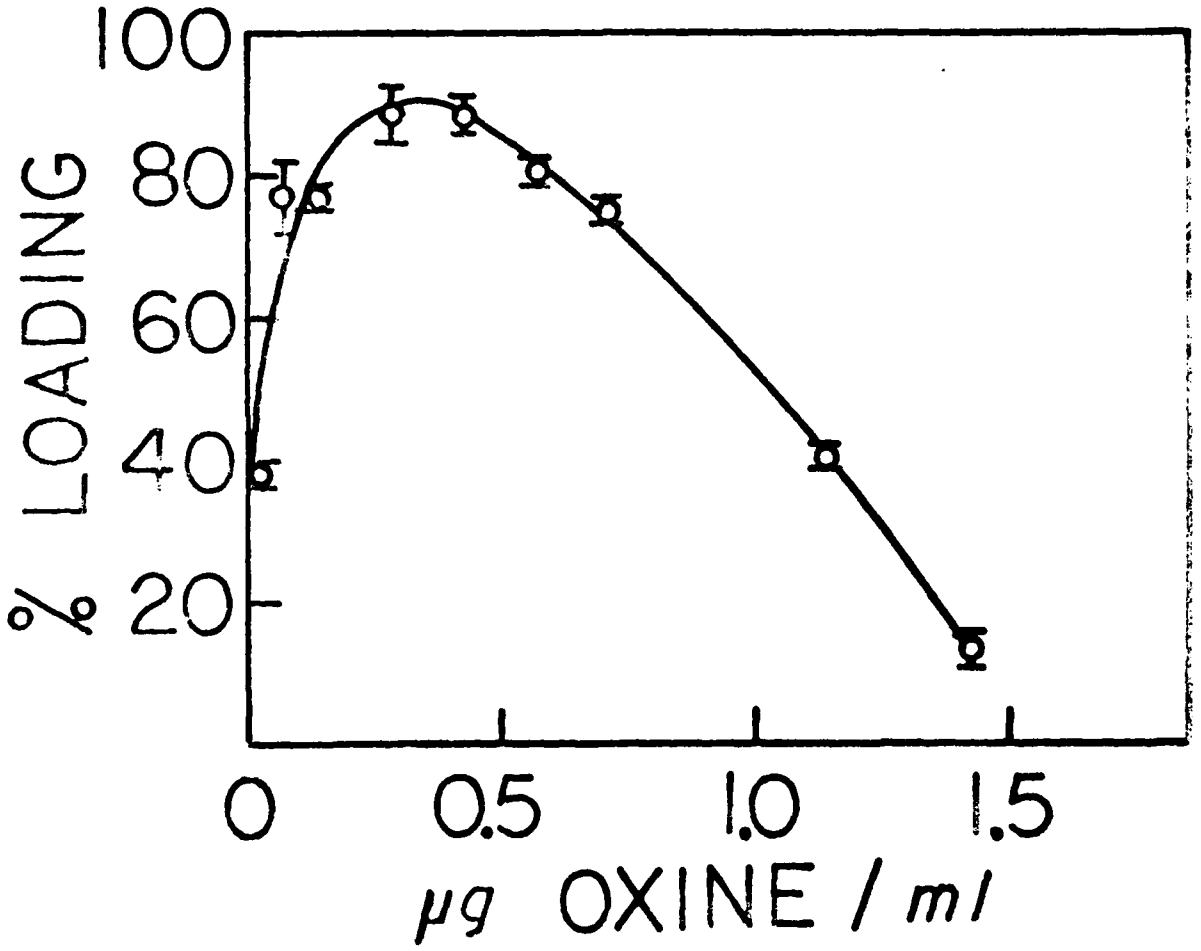


Figure 9. Dependence of the loading efficiency of $^{67}Ga^{3+}$ to liposomes on the concentration of 8-HOQ. The experimental conditions were identical to the one described in Fig. 8. Each point is an average of three measurements.

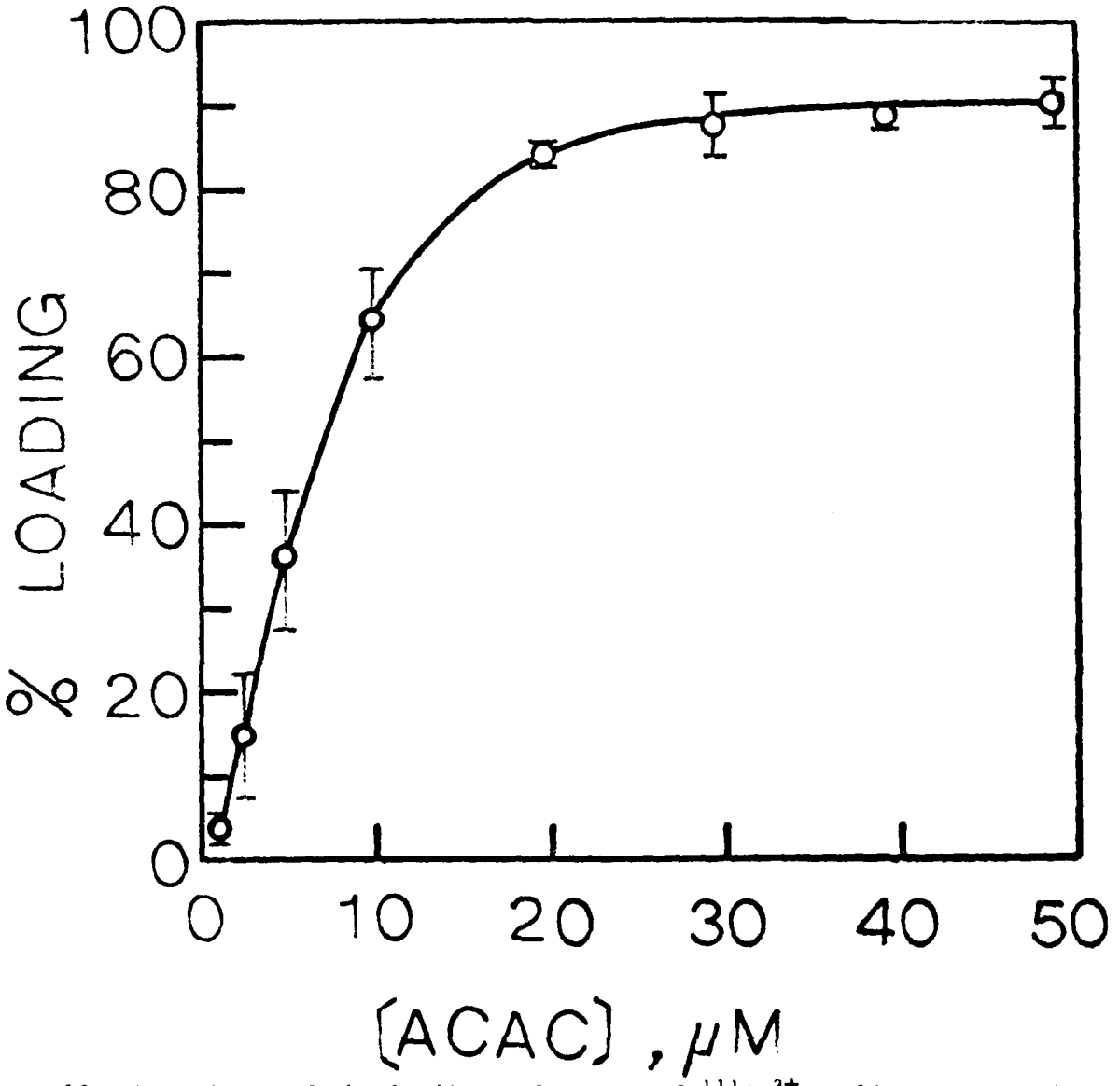
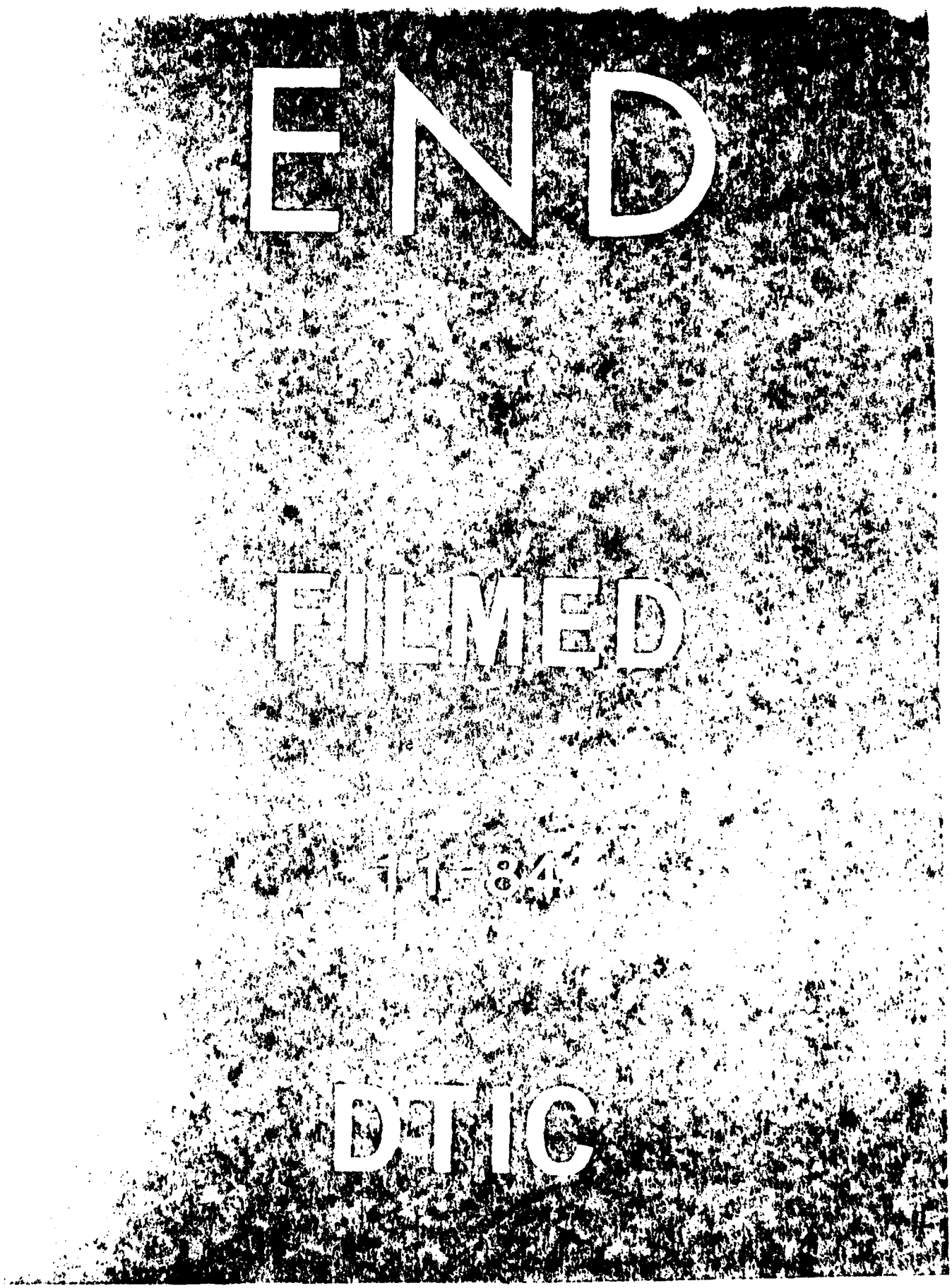


Figure 10. Dependence of the loading efficiency of $^{111}In^{3+}$ to liposomes on the concentration of acetylacetone. The loading was carried out by adding 250 µl of a varying amount of acetylacetone in 0.9% NaCl, 5mM Tris-HCl, pH 7.8 containing 1-10 µ Ci In-111 to 1 ml of small unilamellar SM:CH(2/1;M/M) liposomes in 0.9% NaCl,5mM sodium acetate, pH 5.5. A solution of 0.106 M sodium phosphate, pH 7.4, 1mM NTA was encapsulated in liposomes by probe sonication. The yield of loading was determined as described in Figure 8. Each point is an average of three measurements.

AcAc:	acetylacetone
CH:	cholesterol
DPPC:	L-α-dipalmitoyl phosphatidylcholine
DPPE:	L-α-dipalmitoyl phosphatidylethanolamine
DSPC:	L-α-distearoyl phosphatidylcholine
EDTA:	ethylenediamine-N,N,N',N'-tetraacetic acid
$\langle G_{22}(\infty) \rangle$:	time-integrated perturbation factor. This is the parameter measured by the counters of gamma-ray perturbed angular correlation spectrometer. The value of $\langle G_{22}(\infty) \rangle$ has a range from 0.0 to 1.0. In the system of liposomes, a low value of $\langle G_{22}(\infty) \rangle$ closed to 0.0 means that the liposomes are greatly perturbed and release their encapsulated contents. On the other hand, intact liposomes have a characteristic high $\langle G_{22}(\infty) \rangle$ of about 0.60.
8-HOQ:	8-hydroxyquinoline
8-HOQ-SO_4:	8-hydroxyquinoline sulfate
8-HOQ ($^{111}In^{3+}$):	the complex of 8-HOQ with $^{111}In^{3+}$
MLV:	multilamellar vesicles
NTA:	nitrilotriacetic acid
PAC:	perturbed angular correlation
SM:	bovine brain sphingomyelin
SUV:	small unilamellar vesicles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